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# Evaluation of Combined General Primer-Mediated PCR Sequencing and Type-Specific PCR Strategies for Determination of Human Papillomavirus Genotypes in Cervical Cell Specimens▽

Véronique Fontaine,[1]* Corinne Mascaux,[2] Christine Weyn,[1] Aurore Bernis,[1] Nathalie Celio,[1] Philippe Lefèvre,[1] Leonard Kaufman,[3] and Christian Garbar[2]†

*Laboratory of Molecular Virology, ISP/Institut Pasteur, rue Engeland 642, 1180 Brussels, Belgium[1]; Department of Pathology, CHU Charleroi, Boulevard Zoé Drion 1, 6000 Charleroi, Belgium[2]; and Department of Biostatistics and Medical Informatics, Brussels Free University/VUB, Brussels 1090, Belgium[3]*

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**A strategy combining human papillomavirus general primer (mainly the PGMY primers)-directed PCR sequencing and type-specific PCR is presented. DNA samples were first tested in general primer-mediated PCR. The amplified fragments of positive samples after ethidium bromide-stained DNA gel analysis were further sequenced, and corresponding DNA samples were further analyzed by PCR using type-specific primers for human papillomavirus (HPV) types 16, 18, 31, and 52. The comparison of the results of 157 samples analyzed by this strategy in parallel with the Hybrid Capture 2 tests and with the HPV INNO-LiPA (Innogenetics line probe assay) shows that this method is suitable for HPV detection and genotyping in cervical cell samples. Although the PCR sequencing method is as sensitive as the HPV INNO-LiPA for HPV detection, our method allows the identification of a broader range of HPV types. In contrast, the HPV INNO-LiPA was less time-consuming and better identified coinfections.**

It is now well accepted that all cervical carcinomas contain at least one of the "high-risk" types of human papillomavirus (HPV) DNA (41). Over 100 HPV genotypes have been isolated to date. Among these more than 40 have been shown to infect the genital tract, and 15 of them (genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) have been found to be associated with cervical cancer or high-grade cervical intraepithelial neoplasia and therefore classified as high-risk types (31). In contrast, some HPV genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) are classified as low-risk types (31). Three additional types were classified as probable high risk (26, 53, and 66) while the risk of those previously undetected remains undetermined (31). Differences in geographic distribution of HPV types have been reported to exist between countries (10).

Multiple concurrent and sequential infections with different oncogenic types of HPV are common, usually transient, and disappear spontaneously without clinical lesion. Only persistent high-risk HPV infection induces higher risk for the development of a high-grade precancerous lesion or cervical cancer (14, 41, 43). Since the absence of HPV infection means that the risk of cervical cancer is negligible, testing for HPV has now been incorporated into screening programs that previously relied only on cytology (6). HPV DNA analysis has shown encouraging results when used in conjunction with cytological analysis in primary screening for cervical cancer in women 30 years of age or older (5, 23). As a result of large randomized clinical trials, testing for HPV DNA is now recommended for most women with equivocal findings on cervical cytological analysis (atypical squamous cells of undetermined significance, also called ASCUS) (1, 28). Furthermore, HPV DNA testing provides a unique advantage for early detection of treatment failure (3, 9, 33, 42). Recently, encouraging results with prophylactic HPV vaccines have been reported. These vaccines are believed to be effective against four different HPV types, including the most frequently encountered type, HPV-16. This further emphasizes the need for HPV DNA testing, which will be useful to guide vaccination and to monitor the frequency and the severity of infection by unaffected HPV types (15, 20, 21).

Highly sensitive HPV DNA tests have been developed that rely on molecular biology techniques. For example, the FDA approved the HPV detection kit of Digene/Hybrid Capture 2 (HC2) manufactured by DiGene (MD). Within this test, genotype-specific RNA probes are mixed in a high-risk or a low-risk cocktail, and RNA-DNA hybrids are recognized by an antibody, used both for the capture step and for a signal amplification detection method. This technique fails to discriminate among different genotypes (6). A higher degree of resolution in the identification of genotypes can be achieved using multiple consensus primers and type-specific primers (6, 7, 22, 24, 25). Furthermore, the use of PCR followed by a reverse hybridization line probe assay (LiPA), such as the HPV INNO-LiPA (Innogenetics NV, Ghent, Belgium), has been described as a highly sensitive method to detect and identify multiple infections (25). Here, we have developed and optimized a strategy for HPV detection and genotyping by combining general primer-medi-

* Corresponding author. Mailing address: Laboratory of Molecular Virology, ISP/Institut Pasteur, rue Engeland 642, 1180 Brussels, Belgium. Phone: 32 2 373 33 55. Fax: 32 2 373 32 91. E-mail: vfontaine@pasteur.be.

† Present address: Department of Pathology, AZ-Brussels Free University/VUB, Brussels 1090, Belgium.

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TABLE 1. Primers used for type-specific HPV detection by PCR

| Primer | Sequence | Amplicon length (bp) | Reference or source |
|---|---|---|---|
| HPV-16 V1 | 5′-TTTGGTCTACAACCTCCCCCAGGA-3′ | 105 | This study |
| HPV-16 V2 | 5′-TTCTTTAGGTGCTGGAGGTGTATG-3′ | 105 | This study |
| HPV-18/1 | 5′-CCTTGGACGTAAATTTTTGG-3′ | 115 | 2 |
| HPV-18/2 | 5′-CACGCACACGCTTGGCAGGT-3′ | 115 | 2 |
| HPV-31F | 5′-GTGCTGCAATTGCAAACAGTG-3′ | 200 | This study |
| HPV-31R | 5′-AGGGAGGTGTGGTCAATCC-3′ | 200 | This study |
| HPV-52F | 5′-GCTGAGGTTAAAAAGGAAAGC-3′ | 201 | This study |
| HPV-52R | 5′-ATGCAGACGGTGGTGGGGTAAGG-3′ | 201 | This study |

ated PCR sequencing and type-specific PCR. This approach was compared against commercially available assays.

## MATERIALS AND METHODS

**Collection of cervical samples.** Cervical cells were obtained from a total of 162 women using cervical brushes (Cervexbrush). The cells were resuspended in 20 ml of Easyfix solution (Labonord, Templemars, France). The panel represented a selection of samples from a cross section of cytological diagnoses. Samples were obtained from January 2002 to December 2004 from women attending clinics for routine gynecologic care or during follow-up after treatment at the Department of Gynecology, CHU de Charleroi (Charleroi, Belgium). Samples were stored at 4°C. The laboratory of pathology dispatched an aliquot of each sample both to the laboratory performing the HC2 assay and to the laboratory performing HPV genotyping. The Easyfix cell fixative solutions, containing the Cervexbrush and the cells, were homogenized by mechanical agitation (Vortex) for 30 s before being dispatched (16).

**Cytological diagnosis.** Cytological analyses were performed as previously described (16). We used the Bethesda System 2001 to classify our observations: within the normal limits, ASCUS, and low- and high-grade squamous intraepithelial lesions (Lg-SIL and Hg-SIL, respectively) (32). The HPV detection and genotyping assays were performed blindly without previous knowledge of the cytological analysis results. The Hg-SIL were determined by histopathologic analysis.

**HC2 assay.** The HC2 assays were performed on 5 ml of the residual liquid-based samples. Samples were pelleted at 2,000 rpm for 5 min, and the supernatant was discarded. The cellular pellet was washed once with 1 ml of phosphate-buffered saline, resuspended in 100 μl of cervical sample (Digene Corp., Beltsville, MD), and denatured in an alkaline solution. Classical hybridization, detection, and calibration were made according to the HC2 kit's instructions (27). Probes were used only against the high-risk HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. The HC2 results were expressed as positive or negative depending on the relative light unit of 1 pg/ml of HPV DNA. The HC2 tests were blindly executed by an independent laboratory (Molecular Diagnostic Center, Loverval, Belgium).

**DNA preparation.** Total cellular DNA was extracted by a freeze-thaw method as previously described (30, 38). Briefly, 1 ml of cell suspension was centrifuged (for 10 min at 4°C at 3,000 × *g*), washed with 1 ml of phosphate-buffered saline, further pelleted, and resuspended in 100 μl of 10 mM Tris-HCl, pH 7.5. The suspension was incubated for at least 3 h at −70°C and defrosted for 15 min at 100°C. Cell debris was removed by centrifugation at 14,000 rpm for 30 s. The quality of the extracted DNA was evaluated by PCR using β-globin-specific primers, PC04 (5′-CAACTTCATCCACGTTCACC-3′) and GH20 (5′-GAAGA GCCAAGGACAGGTAC-3′), as described previously (34). Further DNA purification was carried out using a QIAamp blood minikit (QIAGEN) only when no β-globin PCR product could be obtained from the freeze-thaw extraction. Only DNA preparations giving positive amplification after PCR using β-globin primers were further analyzed for HPV detection and genotyping.

**PCR.** Each amplification reaction was carried out in a total volume of 50 μl containing 1.25 units of HotStarTaq DNA polymerase (QIAGEN), 1× PCR buffer provided with the enzyme, and a 200 μM concentration of each deoxynucleoside triphosphate. The concentrations of $MgCl_2$ and primers were optimized for each set of primers. Positive and multiple negative controls were included in all runs. Strict procedures were followed to avoid a false-positive reaction due to contamination. Reagent preparation, DNA extraction, and the addition of positive sample DNA were carried out in separate rooms. Gel electrophoresis and PCR amplification and sequencing were also geographically remote from the pre-PCR preparations. The PCR products were analyzed by electrophoresis on a 2.5% agarose gel stained with ethidium bromide.

**General HPV PCR.** For the PCR using the PGMY primers, the reactions were performed as previously described (18). Briefly, the PCR mixture was complemented with 4 mM $MgCl_2$, 10 pmol of each primer, and 10 μl of DNA preparation. The consensus PCR amplifications were performed in the DNA thermal iCycler (Bio-Rad) because of its ramp modulating capacities. DNA amplification was optimized for the use of the HotStarTaq DNA polymerase in the iCycler. The activation of the enzyme was carried out at 95°C for 9 min, followed by 40 amplification cycles of 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C, with the ramps to 95°C at 2.2°C/s, to 55°C at 2.7°C/s, and to 72°C at 2.2°C/s and a final extension of 7 min at 72°C. The sensitivity assays were performed by amplification of HPV-positive cells (HeLa cells, 10 to 50 copies of HPV-18/cell; SiHa cells, 1 to 2 copies of HPV-16/cell) (29). The nested PCR (nPCR) was performed using GP5+/6+ primers (12): 2 μl of the PGMY PCR product was amplified with 50 pmol of GP5+/6+ primers in a reaction mixture containing 3.5 mM $MgCl_2$; the amplification program was 15 min of activation at 95°C, followed by 40 cycles of 30 s at 94°C, 10 s at 38°C, 30 s at 40°C, and 60 s at 72°C with the ramps to 94°C at 2.2°C/s, to 38°C at 2.7°C/s, to 40°C at 0.2°C/s, and to 72°C at 2.2°C/s and a final extension of 7 min at 72°C.

**Type-specific PCR.** Type-specific detection of HPV-16, -18, -31, and -52 was performed with type-specific PCR primers for each genotype (Table 1). PCR was carried out in the DNA Perkin-Elmer 2400 thermal cycler or in the iCycler

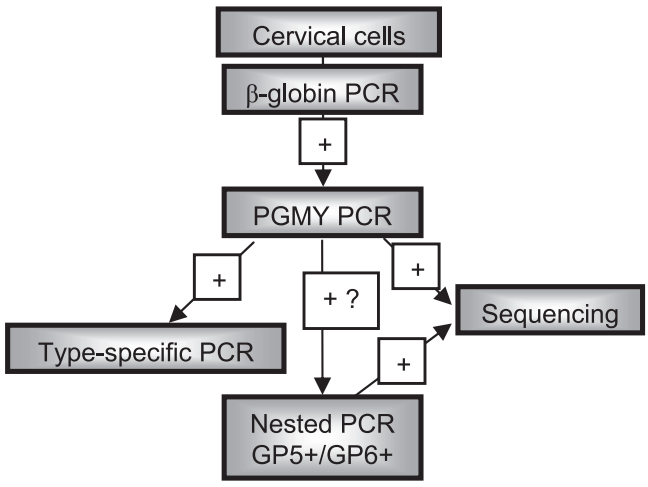


FIG. 1. Strategy for HPV detection and genotyping in cervical cells using PCR sequencing techniques. DNA isolated from cervical scrapes by a freeze-thaw method was subjected to β-globin PCR. Negative samples were further purified on a mini-column, and β-globin-positive samples were analyzed in PGMY PCR. If the PGMY PCR yielded a negative result, the sample was considered HPV negative. Samples with borderline results were retested in nPCR using PGMY as external primers and GP5+/6+ as internal primers. Typing of all the positive samples was carried out by sequencing the amplimers using the PGMY or the GP5+/6+ primers. In parallel, all HPV-positive samples were also tested in HPV-16, -18, -31, and -52 type-specific PCRs.

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TABLE 2. Comparison of HPV detection results obtained by the HC2 assay and the PCR sequencing strategy[a]

| PCR sequencing result | HC2 assay (no. of samples) | | Total no. of samples |
|---|---|---|---|
| | Negative | Positive | |
| Negative | 78 | 5 | 83 |
| Positive | 15 | 53 | 68 |
| Total no. of samples | 93 | 58 | 151 |

[a] The difference between the two methods for the detection of HPV was significant (two-sided McNemar chi-square test for matched pairs, $P = 0.041$). Percent agreement, 86.8; kappa value, 0.729 (0.673–0.785) ($P < 0.001$).

TABLE 4. Comparison of HPV detection results obtained by the INNO-LiPA and the PCR sequencing strategy[a]

| PCR sequencing result | INNO-LiPA (no. of samples) | | Total no. of samples |
|---|---|---|---|
| | Negative | Positive | |
| Negative | 36 | 7 | 43 |
| Positive | 1 | 65 | 66 |
| Total no. samples | 37 | 72 | 109 |

[a] The difference between the two methods for the detection of HPV was not significant (two-sided McNemar chi-square test for matched pairs, $P = 0.070$). Percent agreement, 92.6; kappa value, 0.843.

(Bio-Rad) using 50 pmol of each specific primer and 10 μl of the isolated DNA. For HPV-16 detection, the reactions were performed using the new primers HPV-16 V1 and HPV-16 V2 and 4 mM $MgCl_2$. The PCR conditions were as follows: activation of the HotStarTaq for 15 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 54°C for 45 s, and primer extension at 72°C for 60 s, with a final extension of 7 min at 72°C. For HPV-18 detection, the reactions were performed using the primers HPV-18/1 and HPV-18/2 as described earlier (2) and 2.5 mM $MgCl_2$. The PCR conditions were identical to the HPV-16 PCR except that the annealing step was performed at 51°C for 30 s. For HPV-31 detection, the reactions were performed using the new primers HPV-31F and HPV-31R and 2.5 mM $MgCl_2$. The PCR conditions were activation for 15 min at 95°C, followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 53°C for 60 s, and primer extension at 72°C for 60 s, with a final extension of 7 min at 72°C. For HPV-52 detection, the reactions were performed using the new primers HPV-52F and HPV-52R and 3.5 mM $MgCl_2$. The PCR conditions were identical to HPV-18 PCR except that the annealing temperature was 53°C.

**Sequence analysis.** HPV general PCR products were purified using a GFX column (Amersham) according to the manufacturer's instructions. Direct DNA sequence analysis was performed using a capillary sequencer (ABI Prism 3100) with PGMY09 primer mixture, PGMY11 primer mixture (18), and GP5+ or GP6+ primers (12). When a low-risk HPV was identified using these primers, sequencing of the amplicons was repeated using only selected primers unsuitable for sequencing the identified low-risk HPV.

**SPF10 PCR-LiPA.** The SPF10 PCR-LiPA (Innogenetics, Ghent, Belgium) assay was performed as previously described (25, 26). The current version of the SPF10-LiPA contains probes for high-risk HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68; undetermined-risk HPV genotypes 53, 66, and 70; and low-risk HPV genotypes 6, 11, 40, 42 to 44, 54, and 74. The SPF10 HPV PCR and hybridization steps were carried out following the instructions included with the INNO-LiPA kit. The PCR was optimized for the use of the HotStarTaq DNA polymerase (QIAGEN) in the iCycler as follows: activation of the enzyme was carried out by a 15-min incubation at 95°C, and the amplification was carried out during 40 cycles that included denaturation at 95°C for 30 s, annealing at 52°C for 45 s, and primer extension at 72°C for 45 s, with the ramps to 95°C at 2.7°C/s, to 52°C at 1.5°C/s, and to 72°C at 1.5°C/s; a final extension was carried out for 5 min at 72°C. Ten microliters of the PCR product, containing biotin at the 5′ end of the primers, was denatured in an alkaline solution and incubated under stringent conditions with oligonucleotide probes containing a poly(dT) tail immobilized in parallel lines on nitrocellulose membrane strips. After stringent washing, the hybrids were detected by alkaline phosphatase-streptavidin conjugate and the substrates 55-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium, resulting in a purple precipitate at the positive probe lines.

TABLE 3. Comparison of HPV detection results obtained by the INNO-LiPA and the HC2 assay[a]

| HC2 assay result | INNO-LiPA (no. of samples) | | Total no. of samples |
|---|---|---|---|
| | Negative | Positive | |
| Negative | 34 | 21 | 55 |
| Positive | 3 | 48 | 51 |
| Total no. of samples | 37 | 69 | 106 |

[a] The difference between the two methods for the detection of HPV was significant (two-sided McNemar chi-square test for matched pairs, $P < 0.001$). Percent agreement, 77.4; kappa value, 0.552.

**Statistics.** Statistical analyses were performed using SPSS, version 11.0, for Windows. Statistical tests were performed two sided at the 5% level of significance. The kappa statistic was calculated to evaluate the agreement between HPV detection methods (13). In general, a kappa value above 0.75 indicates excellent agreement, between 0.4 and 0.75 indicates fair to good agreement, and below 0.4 represents poor agreement. Significance testing for the unequal distribution of discordant results was performed by McNemar's chi-square test for matched pairs when dichotomous outcomes are compared (4). The number of types detected per sample by each genotyping strategy was compared using the Wilcoxon signed-rank test.

## RESULTS

Analyses were performed stepwise (Fig. 1). First, the quality of the DNA preparation was assessed by PCR using β-globin primers. With five exceptions (3.0%), all DNA preparations were β-globin positive and thus adequate for further analysis. Among the 157 DNA samples suitable for HPV analysis, 70 (44.6%) corresponded to a negative cytological analysis, 36 (23.0%) corresponded to an ASCUS, 32 (20.4%) corresponded to a low-grade cervical intraepithelial neoplasia, 18 (11.4%) corresponded to a high-grade cervical intraepithelial neoplasia, and 1 (0.6%) corresponded to a cervical adenocarcinoma. DNA from clinical samples was then assessed first by PCR using the very sensitive HPV PGMY09/11 general primers, a more recent version of the MY09/MY11 primers (11). DNA giving equivocal results was further assessed by nPCR. In the nPCR, the first run of PCR was performed using the general PGMY primers, and the second run was performed by using the GP5+/6+ consensus primers and 2 μl of the PCR products of the first run. The nPCR was used for only 3 samples of 157 samples (1.9%) and gave us the identification only of undetermined-risk HPV (homologous HPV-62, HPV-62, and homologous HPV-91) and possibly low-risk types according to phylogenetic studies (8, 36). The amplicons from the general PCR, detected by agarose gel electrophoresis and ethidium bromide staining, were further analyzed by sequencing in order to identify at least one HPV genotype per positive sample. DNA from clinical samples that were positive after PCR using HPV general primers was also further tested in PCR using HPV type-specific primers (Table 1). The ability of the newly designed type-specific HPV primers to amplify specific viral DNA was tested using DNA preparations of SiHa cells (1 to 2 copies of HPV-16/cell), HeLa cells (10 to 50 copies of

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TABLE 5. Distribution of the HPV detection results obtained by the INNO-LiPA, the PCR sequencing strategy, and the HC2 assay and correlation with cytological analysis

| Cytology[a] | HPV detection result by indicated method (no. of samples [%])[b] | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | INNO-LiPA | | | HC2 | | | PCR sequencing | | |
| | − | + | *n* | − | + | *n* | − | + | *n* |
| WNL | 20 (56%) | 16 (44) | 36 (100) | 57 (86) | 9 (14) | 66 (100) | 56 (80) | 14 (20) | 70 (100) |
| ASCUS | 9 (35%) | 17 (65) | 26 (100) | 22 (63) | 13 (37) | 35 (100) | 19 (53) | 17 (47) | 36 (100) |
| Lg-SIL | 8 (28%) | 21 (72) | 29 (100) | 12 (37) | 20 (63) | 32 (100) | 11 (34) | 21 (66) | 32 (100) |
| Hg-SIL | 0 (0%) | 17 (100) | 17 (100) | 2 (11) | 16 (89) | 18 (100) | 0 (0) | 18 (100) | 18 (100) |

[a] WNL, within normal limits.
[b] *n*, no. of samples.

HPV-18/cell), and patient samples known to contain HPV-31 or HPV-52 DNA.

Type-specific PCR allowed us to achieve HPV genotyping of clinical samples despite sequencing difficulties and to identify coinfections (15 samples out of 92, or 16%). Type-specific PCR improved HPV-16 (9 out of 24 genotyped), HPV-52 (7 out of 12 genotyped), HPV-31 (6 out of 15 genotyped), and HPV-18 (2 out of 15 genotyped) detection. For sequencing, the use of a subset of primers of the 18 PGMY primers to avoid repeated detection of low-risk HPV allowed us to identify a coinfection in 2 samples out of 92 (2%).

The results obtained using different techniques, the PCR sequencing strategy, hybridization (HC2 from Digene), or PCR hybridization (INNO-LiPA HPV genotyping from Innogenetics), are summarized in Tables 2, 3, and 4.

The agreement between the results obtained by the HC2 assays and the PCR sequencing strategy to detect high-risk HPV (Table 2) was high (86.8%; $\kappa = 0.729$). However, a higher percentage of samples was scored HPV positive with the PCR sequencing strategy than with the HC2 assay (McNemar's test, $P = 0.041$). Among the PCR sequencing-positive, HC2-negative samples ($n = 15$), two corresponded to cells from the Hg-SIL profile by cytological analysis (Table 5), suggesting a lower sensitivity of the HC2 assay.

The percentage of agreement was reduced when we compared data from the HC2 assay and the INNO-LiPA (77.4%; $\kappa = 0.552$) (Table 3). The HPV-positive score was statistically much higher in the INNO-LiPA than in the HC2 assay (McNemar's test, $P < 0.001$).

Interestingly, the percentage of agreement was the highest (92.6%; $\kappa = 0.843$) when the results obtained by the INNO-LiPA and the PCR sequencing strategy were compared (Table 4). Although the INNO-LiPA was slightly more sensitive than the PCR sequencing strategy as it detected seven more positive samples (6%), the difference was not statistically significant (McNemar's test, $P = 0.070$).

Overall, the percentages of agreement between the three methods, summarized in Fig. 2, suggested that the results obtained by the HC2 assay are more often discordant (14% instead of 1% for PCR and sequencing and 7% for the INNO-LiPA).

When we compared the genotyping results obtained by either the PCR sequencing strategy or the INNO-LiPA (Table 6), we observed that multiple infections were most often detected with the INNO-LiPA. Indeed, 50% of the multiple infections detected by the INNO-LiPA corresponded to an infection caused by more than two HPV types, while the PCR sequencing strategy never succeeded in identifying more than two HPV types in one clinical sample. The number of types detected per samples was 1.94 ± 1.381 in the INNO-LiPA and 1.18 ± 0.414 in the PCR sequencing strategy, statistically significant (asymptotic significance [2-tailed], $P < 0.001$) according to the Wilcoxon rank test.

Among the HPV types that were detected in this study, HPV-16 was the most prevalent, followed by HPV-31 and HPV-52 (Fig. 3). The agreement for HPV-16 genotyping was

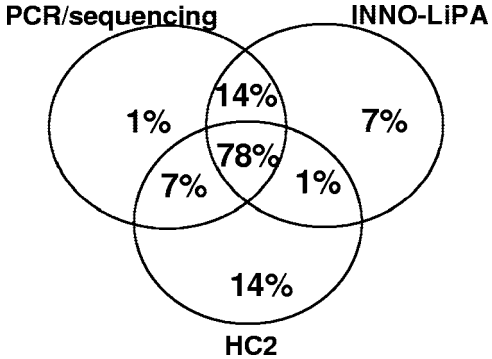


FIG. 2. Mathematic representation of the distribution of the HPV detection results obtained for 107 samples using three different methods, PCR sequencing strategy, HC2 assay, and HPV INNO-LiPA. Overlapping sections between circles represent identical results (positive and negative) obtained with two or three methods.

TABLE 6. Comparison of HPV multiple-infection genotyping results obtained by the INNO-LIPA and the PCR sequencing strategy[a]

| PCR sequencing result | INNO-LiPA result (no. of samples) | | Total no. of samples |
|---|---|---|---|
| | Simple infection | Multiple infection | |
| Simple infection | 45 | 20 | 65 |
| Multiple infection | 0 | 15 | 15 |
| Total | 45 | 35 | 80 |

[a] The difference between the two methods for the detection of multiple infection was significant (two-sided McNemar chi-square test for matched pairs, $P < 0.001$). Percent agreement, 75; kappa value, 0458.

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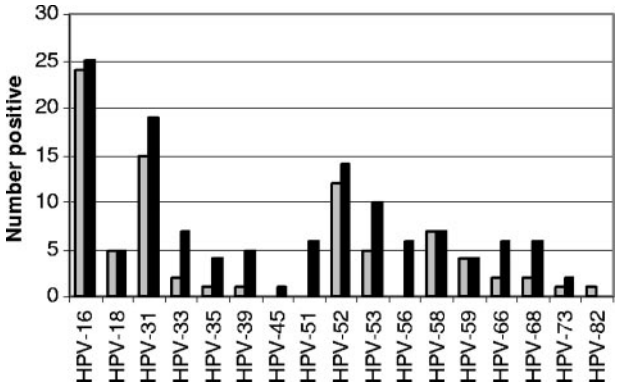


FIG. 3. Distribution of high-risk and probable high-risk HPV genotypes according to the PCR sequencing strategy (gray) or to the HPV INNO-LiPA (black) among the samples tested by both methods. The total numbers of positive results by HPV type are plotted. The type-specific results include positive results for HPV types from specimens infected with both single and multiple HPV types.

high between the two methods (96.4%; with McNemar's test, $P = 1.000$).

Remarkably, among the single-case infections ($n = 49$), the same genotype was identified by both methods in 46 of 49 samples (93.9%). The discordant results were comprised of one HPV-84 and two HPV-91 identifications by the sequencing method, corresponding to HPV-39, HPV-44, and HPV-66 as determined by the HPV INNO-LiPA method. These discrepancies could be due to coinfections by the two detected HPV types present in limited quantities in the analyzed samples.

## DISCUSSION

In this study, a PCR sequencing method was combined with type-specific PCR to detect HPV infection in cervical cells. Our primary screening was based on PCRs using the PGMY09/11 primers (18). The PCR conditions were adapted and optimized for the use of a different *Taq* DNA polymerase (QIAGEN) and for a different DNA thermocycler (iCycler from Bio-Rad). Indeed, as previously reported, adapting the rate of temperature changes in the thermocycler is essential for optimization of the sensitivity and also specificity of the general primer-mediated PCR (19, 39). To detect HPV by general primer-mediated PCR, we therefore preferred to use a thermocycler with ramping flexibility. To achieve a maximal sensitivity for detecting HPV-16, -18, -31, and -52, frequently found in the Belgian population, we designed type-specific primers to generate amplicons of relatively short length (100 to 200 bp). The combination of broad-spectrum and type-specific PCR allowed us to achieve a high sensitivity of detection for these four human papillomavirus types, especially for HPV-16 and HPV-18, compared to the INNO-LiPA assay. In the case of multiple infections, adding type-specific PCRs to our strategy allowed us to partially circumvent probable competition between types for primers annealing during PCR and sequencing reactions, as often observed in HPV-31 and HPV-52 infections. Recently, the combination of broad-spectrum (SPF10) and type-specific PCR has been shown to result in an even more accurate method (40).

The PCR sequencing approach was compared to the commercially available high-risk HPV detection HC2 assay (Digene) and to the HPV genotyping INNO-LiPA (Innogenetics). The concordance between the PCR sequencing strategy, the INNO-LiPA, and the HC2 assay was high, and agreement was very good or nearly very good ($\kappa = 0.843$ or $\kappa = 0.729$, respectively). Overall, the INNO-LiPA proved to be more sensitive. In the amplification of SiHa cell DNA, PGMY PCR was positive using 100 pg of genomic DNA, as previously reported (17). This sensitivity is at least 10 times higher when other general HPV primers such as the CPI/IIG or the GP5+/6+ are used (7, 37; V. Fontaine, unpublished data). The SPF10 PCR (HPV INNO-LiPA; Innogenetics) and the nPCR proved to be even more sensitive, as previously reported (17, 22). nPCR was, however, impractical because of its higher risk of contamination and, hence, was utilized only to confirm ambiguous PGMY PCR results. The 60 bp amplified by the SPF10 primers are probably easier to obtain than the large fragments of 450 bp amplified with the PGMY primers (18). The efficiency of the primer pair has, indeed, been inversely correlated to the length of the amplimer (2).

SPF10 PCR had the highest sensitivity and was able to identify 38% or 16% HPV positivity of otherwise HPV-negative samples as determined by the HC2 or the PCR sequencing method, respectively. Most types found were included in the HC2 probe cocktail; only 3 of 18 corresponded to types not found in the cocktails (one HPV-53 and two HPV-66), and all could potentially be detected by the PCR sequencing strategy. Multiple infections were especially more often detected by the INNO-LiPA. Recently, a restriction fragment length polymorphism typing method using the *Hpy*CH4V enzyme has been described as an easy tool to discriminate between single or multiple infections (35). This strategy could be of use to improve the PCR sequencing strategy by orienting type-specific PCR and sequencing analysis. Overall, the HPV detection rate increased with the severity of the cytological diagnosis (Table 5). Of note, two negative results obtained by the HC2 assay for samples corresponded to an Hg-SIL profile, known to be always associated with HPV.

Using our PCR sequencing strategy, some uncommon HPV genotypes could be identified (HPV-62, -69, -82, and -84; hom62 and hom91) in contrast with the HC2 assay and the HPV INNO-LiPA, which are probe-restricted assays. HPV-82 is considered a high-risk HPV while the others are of undetermined risk. All were detected in cells with a normal cytology profile except for HPV-69, closely related to the probable high-risk HPV-26 in phylogenetic classification and identified in cells with a low-grade intraepithelial cell neoplasia (8). Because of the larger number of HPV genotypes identified, the PCR sequencing techniques may be useful in a research setting. This emphasizes that the involvement of certain HPV types in various lesions could be underestimated if the method used for HPV detection and identification does not cover a broad spectrum of types.

In conclusion, our results underline some advantages of the HPV INNO-LiPA over the PCR sequencing strategy. The INNO-LiPA had a higher degree of sensitivity, was more rapid, and was capable of identifying more multiple infections. Nonetheless, the PCR sequencing strategy allowed the identification of a broader range of HPV types due to the wide spectrum of

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the PGMY PCR combined with the unrestricted capacity of the sequencing method.

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